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ORIGINAL ARTICLE

The *KIT* D816V expressed allele burden for diagnosis and disease monitoring of systemic mastocytosis

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Abstract The activating *KIT* D816V mutation plays a central role in the pathogenesis, diagnosis, and targeted treatment of systemic mastocytosis (SM). For improved and reliable identification of *KIT* D816V, we have developed an allele-specific quantitative real-time PCR (RQ-PCR) with an enhanced sensitivity of 0.01–0.1 %, which was superior to denaturing high-performance liquid chromatography (0.5–1 %) or conventional sequencing (10–20 %). Overall, *KIT* D816 mutations were identified in 146/147 (99 %) of patients (D816V, $n=142$; D816H, $n=2$; D816Y, $n=2$) with SM, including indolent SM (ISM, $n=63$, 43 %), smoldering SM ($n=8$, 5 %), SM with associated hematological non-mast cell lineage disease (SM-AHNMD, $n=16$, 11 %), and aggressive SM/mast cell leukemia±AHNMD (ASM/MCL, $n=60$, 41 %). If positive in BM, the *KIT* D816V mutation was found in PB of all patients with advanced SM (SM-AHNMD, ASM, and MCL) and in 46 % (23/50) of patients with ISM. There was a strong correlation between the *KIT* D816V expressed allele burden (*KIT* D816V EAB) with results obtained from DNA by genomic allele-specific PCR and also with disease activity (e.g., serum tryptase level), disease subtype (e.g., indolent vs. advanced SM) and survival. In terms of monitoring of residual disease, qualitative and quantitative assessment of *KIT* D816V and *KIT* D816V EAB was successfully used for sequential analysis after chemotherapy or allogeneic stem cell transplantation. We therefore conclude that RQ-PCR assays for *KIT* D816V are useful complimentary tools for diagnosis, disease monitoring, and evaluation of prognosis in patients with SM.

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Introduction

Systemic mastocytosis (SM) is characterized by abnormal proliferation and accumulation of mast cells in diverse tissues such as bone marrow (BM), skin, liver, spleen, or gastrointestinal tract. Diagnosis is based on the presence of one major (multifocal dense infiltrates of mast cells in BM biopsies and/or in sections of other extracutaneous organs) and at least one minor, or the presence of three out of four minor criteria (>25 % atypical cells on BM smears or spindle-shaped mast cell infiltrates, the presence of a mutation in the tyrosine kinase (TK) KIT in BM or other extracutaneous organs, expression of CD2 and/or CD25 by mast cells in BM, peripheral blood (PB) or another extracutaneous organ and baseline serum tryptase concentration >20 μg/l) [1–4].

Diagnosis of aggressive SM (ASM) is based on the presence of one or more C-findings [cytopenia (ANC$<1\times10^9$/l, Hb<10 g/dl, or platelets$<100\times10^9$/l), hepatomegaly with impaired liver function, palpable splenomegaly with signs of hypersplenism, malabsorption with significant hypoalbuminemia, significant weight loss >10 % over the last 6 months and/or large osteolyses). Mast cell leukemia (MCL) is defined by the presence of at least 20 % mast cells on BM smears. Organ damage, C-findings and markedly elevated STL >200 μg/l are frequently observed [5–7]. In a subset of patients, an associated clonal hematologic non-mast cell disorder is present (SM-AHNMD), most frequently chronic myelomonocytic leukemia (SM-CMML) or hypereosinophilic syndrome/chronic eosinophilic leukemia (SM-HES/CEL). Smoldering SM (SSM) is a recently defined subgroup, which requires at least two of three B findings (hepatomegaly and/or splenomegaly, BM mast cells >30 %, STL >200 μg/l, increased marrow cellularity/dysplasia or organomegaly) in the absence of C findings. Patients with typical BM infiltrates but not fulfilling diagnostic criteria for ASM, MCL, SM-AHNMD, or SSM are diagnosed as indolent SM (ISM). Median survival for SM-AHNMD, ASM/MCL, SSM, and ISM is 24, 41, 120, and 198 months, respectively [8–10].

Depending on subtype, adequate material, and assay sensitivity, a gain of function mutation from aspartate to valine at codon 816 within the TK domain of KIT (*KIT* D816V), the receptor for stem cell factor, can be identified in cells from BM or other affected tissues in >80 % of patients [10–12]. Very rarely, other somatic (D815K, D816F, D816H, D816Y, and V560G) and germline (F522C, A533D, K509I, and del419) *KIT* mutations have been reported [10, 13]. However, accurate numbers on the overall frequency of *KIT* D816V in the diverse SM subtypes remain largely unknown because of (1) the rarity of disease, (2) highly variable mast cell burden in BM and PB, and (3) inadequate sensitivity of disparate assays. Conventional sequencing (CS) has a sensitivity of only 10–20 % and repeatedly fails to detect the mutation in those patients in which only a small fraction of cells is affected. Because mast cells are usually not present in PB, the use of BM is recommended for diagnostic procedures. However, *KIT* mutations may not be restricted to mast cells and may thus also be found in other non-mast cell lineages suggesting that the expansion of clonal mast cells derives from an uncommitted hematopoietic stem cell [14–16].

Using a highly sensitive allele-specific quantitative real-time PCR (RQ-PCR), we here demonstrate that the *KIT* D816V mutation is present in more than 97 % of SM patients. If positive in BM, it is found in PB from all patients with advanced SM (SM-AHNMD, ASM/MCL) and in 46 % of ISM patients. There is a strong correlation between the *KIT* D816V expressed allele burden (*KIT* D816V EAB) and disease activity, disease subtype, and consequently also survival. In addition, serial measurement of the *KIT* D816V EAB is useful for monitoring of residual disease after chemotherapy or allografting.

Materials and methods

Patients, samples, and cell lines

Overall, 210 samples from 147 patients (male, $n=74$; female, $n=73$; median age, 59 years; range, 26–82) with SM [ISM, $n=63$ (43 %); SSM, $n=8$ (5 %); SM-AHNMD, $n=16$ (11 %, sAML, $n=2$; MDS/MPN-u, $n=2$; MPN, $n=1$; CEL/HES, $n=9$; CMML, $n=2$); ASM/MCL±AHNMD, $n=60$ (41 %)] according to the World Health Organization 2008 diagnostic criteria were studied (Table 1). CML patients in major molecular remission ($n=65$) and healthy volunteers ($n=20$) served as controls. In 62 patients, samples were collected contemporaneously from BM and PB (ISM, $n=28$; SSM, $n=4$; SM-AHNMD, $n=5$; ASM/MCL, $n=25$). All patients gave informed consent according to the Declaration of Helsinki. The study was approved by the institutional review board as part of the "German Registry on Eosinophilia and Mastocytosis." For assay sensitivity calculation, cell dilutions of D816V positive HMC-1.2 cell line (kindly provided by Dr. Butterfield, Mayo Clinic Rochester, MN, USA) [17] in *KIT* wild-type NB4 cells (DSMZ Braunschweig, Germany) and diluted patient RNA in healthy control BM RNA were performed (Clontech, Mountain View, CA, USA).

RNA extraction and cDNA synthesis

Total RNA was extracted using the commercial available TRIzol Reagent (Life Technologies, Darmstadt, Germany) or the CsCl gradient centrifugation method as previously

Table 1 Patients' characteristics

No.	Disease stage	Gender male/female	Age in years median (range)	Number of samples BM infiltration in % median (range)	Number of samples STL (μg/l) median (range)	Analyzed samples		
						n (%)	*n* (%)	Material
63	ISM	24/39	48 (28–76)	*n*=56	*n*=53	91 (43 %)	41 (49 %)	BM
				10 (0–40)	42 (5–229)		50 (39 %)	PB
8	SSM	6/2	50 (44–70)	*n*=8	*n*=61	12 (6 %)	5 (6 %)	BM
				35 (20–50)	68 (35–545)		7 (6 %)	PB
16	SM-AHNMD	6/10	69 (26–82)	*n*=12	*n*=13	21 (10 %)	5 (6 %)	BM
				20 (5–20)	145 (30–402)		16 (13 %)	PB
60	ASM/MCL ±AHNMD	38/22	69 (44–83)	*n*=47	*n*=46	94 (45 %)	32 (39 %)	BM
				50 (5–95)	265 (25–1854)		54 (43 %)	PB
147	Total	74/73	59 (26–83)	*n*=123	*n*=118	210 (100 %)	83 (100 %)	BM
				20 (0–95)	92 (5–1854)		127 (100 %)	PB

ISM indolent systemic mastocytosis (SM), *SSM* smoldering SM, *SM-AHNMD* SM with associated clonal hematologic nonmast-cell lineage disorder, *ASM* aggressive SM, *MCL* mast cell leukemia, *BM* bone marrow, *PB* peripheral blood, *STL* serum tryptase level

described [18]. Complementary DNA (cDNA) was synthesized using random hexamers and MMLV reverse transcriptase (Life Technologies).

RQ-PCR and D-HPLC

Two RQ-PCR assays amplifying both the mutant *KIT* D816V and the *KIT* wild-type (wt) allele were designed (for primer sequences see Supplementary Information Table 1). The total *KIT* assay (*KIT* D816V plus *KIT* wt) served as internal control for cDNA quantity in addition to a previously described *ABL* control gene assay [19]. PCR was performed using the universal "mastermix" (LightCycler Faststart plus set hybridization probes, Roche Diagnostics, Mannheim, Germany) on a LightCycler instrument 1.5 (Roche Diagnostics) in a final volume of 20 μl with 2 μl cDNA or plasmid product (500 nm primer; 250 nm probes). RQ-PCR cycling conditions were as follows: 95 °C (10 min), 45 cycles: 95 °C (1 s), 60 °C (10 s), and 72 °C (26 s). A plasmid standard curve was used for absolute quantification. [Supplementary Information (Fig. 1, Methods)]. *KIT* D816V EAB was calculated as ratio between mutant *KIT* D816V and total *KIT* transcripts (mutant *KIT*+wt *KIT* expressed as % *KIT* D816V/*KIT*). In order to validate the novel *KIT* D816V EAB PCR assay, samples and results were compared to a recently published RQ-PCR on DNA level [20].

For denaturing high-performance liquid chromatography (DHPLC), *KIT* was amplified by nested RT-PCR [Supplementary Information (Table 1, Methods)] using the high fidelity Optimase Polymerase (Transgenomic, Omaha, NE, USA). *KIT* amplicons were analyzed after heteroduplex formation at 58 °C using a Wave 3500HT System (Transgenomic). Equal amounts of PCR product and an estimated 1 % mutant (diluted HMC1 cell line) were analyzed to confirm low mutant or high mutant proportions of the *KIT* D816V mutation. PCR products were sequenced bidirectional using the amplification primers.

Statistical analysis

Correlation between variables was investigated by Spearman's rank correlation coefficient, scatter plots or Mann–Whitney *U* test (*t*-approximation). Survival analysis was calculated using the Kaplan–Meier method. Significance level was 0.05 for all statistical testing. Analyses were performed with Graph pad prism 5.

Results

Assay sensitivity of RQ-PCR and D-HPLC

The maximum sensitivity of RQ-PCR and D-HPLC was calculated by analyzing serial cell dilutions of *KIT* D816V-positive HMC1 cells in NB4 cells and total RNA dilutions from *KIT* D816V-positive patients (*KIT* D816V EAB 50 and 80 %) with total RNA from BM of healthy individuals. The D-HPLC was optimized for high sensitivity detection of the most common *KIT* D816V mutation. D-HPLC detected mutant *KIT* D816V down to 0.5–1 % of mutant in normal cells based on cell line and RNA dilutions. For the RQ-PCR assay, enhanced sensitivity of 0.01–0.1 % mutated cells assessed by cell line dilutions could be achieved. However the *KIT* D816V EAB of the analyzed samples in the present data set was higher than 0.01 % in all cases and typically achieved minimal values of 0.1 %. The inter- and intra-assay variations were calculated by repeated analyses (eight and ten times tested) of a cDNA cell line dilution of the *KIT* D816V-positive HMC1 cell line with the *KIT* wt NB4 cell line

corresponding to a mutation ratio of 0.1 and 25 %. The levels of assay variation were high in the samples with low mutation levels (11 and 35 %) and low in the samples with high mutation levels (9 vs. 15 %).

The RQ-PCR assay for *KIT* D816V and total *KIT* showed linearity over five orders of magnitude (from 40 to 4,000,000) as assigned by standard plasmid dilutions. In order to analyze the nonspecific cross-reaction of the wt allele with the *KIT* D816V mutation-specific assay, cDNA samples from 85 *KIT* D816V negative healthy volunteers ($n=20$) and CML patients in major molecular remission (*BCR-ABL*/*ABL* ratio <0.1 %, n=65) were analyzed. A nonspecific cross amplification (≥36CP) was observed in 34 of 85 (40 %) samples by RQ-PCR. We therefore assigned samples with less than ≥35CP by RQ-PCR as negative. Analyzing the same cell and mRNA dilutions by CS, an assay sensitivity of only 10–15 % mutant proportion could be achieved (Supplementary Information Fig. 1).

Comparison between CS, D-HPLC, and RQ-PCR

The three assays were applied to 210 samples from 147 SM patients with various SM subtypes. BM samples were available from 83 of 147 (56 %) and PB samples from 127 of 147 (86 %) patients. Four patients (ASM/MCL, $n=2$; ISM, $n=2$) were negative for *KIT* D816V but positive for rarer mutations at codon 816 (D816Y and D816H). These patients had an abnormal elution profile in the D-HPLC assay and the *KIT* D816H/Y mutations were detected by CS (Supplementary Information Fig. 1). One patient with SM-HES/CEL was negative for the *KIT* D816V or other rarer *KIT* D816 mutations in BM and PB using all available techniques. Diagnosis in this patient was defined by mast cell aggregates and immunohistochemistry (positivity for CD25). These five patients were consequently excluded from further analysis of *KIT* D816V expression levels in different disease subtypes of SM. RQ-PCR was the most sensitive technique and identified the *KIT* D816V mutation irrespective of disease subtype in 100 % of BM samples and 78 % of PB samples. All patients were *KIT* D816V-positive in BM. In PB, 46 % of ISM patients were *KIT* D816V positive (Fig. 1). For detection of *KIT* D816V, the overall sensitivities of CS (BM, 74 %; PB, 64 %) and D-HPLC (BM, 99 %; PB, 65 %) were inferior to RQ-PCR (BM, 100 %; PB, 78 %).

Comparison RNA/cDNA vs. DNA

We compared the *KIT* D816V EAB by quantitative RT-PCR with a recently established allele-specific quantitative PCR on genomic DNA [20]. In 25 samples with *KIT* D816V EAB ranging between 0 and 54 %, there was a strong correlation between both assays (Spearman $r=0.899$, $p<0.001$). In an additional 12 patients who were *KIT* D816V-positive in BM but negative in PB by quantitative RT-PCR, 3 patients were also tested negative by genomic PCR, whereas 9 patients were tested positive by genomic PCR. The median genomic allele burden in those nine patients was 0.067 % (range, 0.007–0.13 %), thus confirming the 0.1–0.01 % sensitivity threshold of quantitative RT-PCR.

KIT D816V EAB in BM and PB

In ASM/MCL±AHNMD, the median *KIT* D816V EAB did not significantly differ between BM and PB (32 vs. 37 %; range, 1–99 vs. 0.2–74; p=n.s.), whereas the median *KIT* D816V EAB in ISM/SSM was significantly higher in BM as compared to PB [9 vs. 0.2 %; range, 1.1–50 vs. 0–43; $p<0.0001$ (Fig. 1)]. This was confirmed in contemporaneously collected BM/PB samples of 63 patients (ASM/MCL± AHNMD: BM 27 vs. PB 33 %, p=n.s.; ISM/SSM: BM 9 % vs. PB 0.1 %, $p<0.0001$, data not shown).

Correlation with complementary laboratory parameters and clinical findings

KIT D816V EAB in PB clearly distinguished between indolent and advanced phase disease (Fig. 1). Within the ASM/MCL group, statistically significant different *KIT* D816V EAB levels were observed for patients with or without AHNMD (38 vs. 22 %, $p=0.019$) with or without monocytosis $>1\times10^9$/l (45 vs. 29 %, $p=0.0002$) and with or without elevated serum levels of alkaline phosphatase and gamma glutamyl transpeptidase >2× upper limits of normal, 33 vs. 11 %, $p=0.0079$; data not shown). No differences were found regarding eosinophilia. Seven patients with advanced SM had a *KIT* D816V EAB <5 %. A screen in three of these patients revealed mutations in additional genes (*KIT-ASXL1-CBL-EZH2-TET2*, *KIT-RUNX1*, and *KIT-U2AF1-DNMT3A*) consistent with advanced disease [21, 22]. In ISM, nine patients had a *KIT* D816V EAB ≥5 %. Five of these patients had signs of more advanced disease, e.g., borderline Hb level <12 g/dl ($n=3$), thrombocytopenia <140 x 10^9/l ($n=2$), monocytosis $>1.0\times10^9$/l ($n=2$) or eosinophilia $>1.0\times10^9$/l ($n=1$) without clear diagnosis of AHNMD in BM. In three of the nine ISM patients, comprehensive mutational profiling was performed, which showed no additional mutations apart from *KIT* D816V [21]. Overall, there was a good correlation ($r=0.59$, $p<0.0001$) between *KIT* D816V EAB and STL (Fig. 2). No correlation was observed between BM mast cell infiltration and *KIT* D816V EAB, but there was neither a correlation between BM mast cell infiltration and STL.

Monitoring of residual disease

A significant reduction of the *KIT* D816V EAB in BM and PB was observed in four ASM/MCL±AHNMD patients after

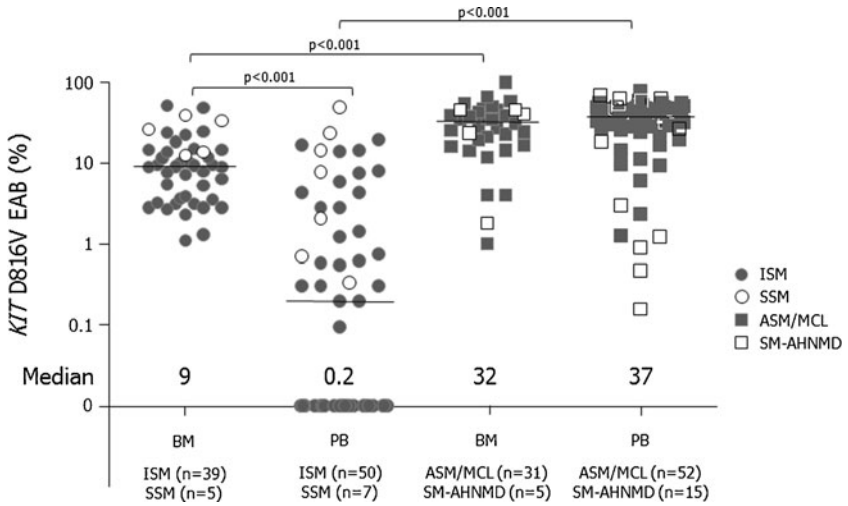

Fig. 1 *KIT* D816V expressed allele burden (EAB) in SM-subtypes: frequency of *KIT* D816V positivity according to disease stage and sample material. Significant differences of the *KIT* D816V EAB in PB and BM were observed for indolent (ISM/SSM) vs. advanced SM (ASM/MCL± AHNMD)

chemotherapy with cladribine ($n=1$), aggressive chemotherapy ($n=1$), or allogeneic stem cell transplantation (SCT, $n=2$), respectively. In the cladribine-treated patient, the *KIT* D816V EAB decreased from 42 to 5 % within 9 months. The mast cell infiltration in BM correspondingly decreased from 80 to 10 %. At the time of relapse (+18 months), the patient presented with progressive organomegaly, thrombocytopenia, and a *KIT* D816V EAB of 35 %. The results were closely mirrored by decrease and increase of STL (Fig. 3). The second patient showed a history of SM for 24 months, when aggressive chemotherapy had to be initiated after progression to secondary acute leukemia. After 4 weeks, the *KIT* D816V EAB decreased from 59 to 22 % (data not shown). Prior to allogeneic SCT of two ASM/MCL patients, the *KIT* D816V EAB in PB was 35 and 45 %, respectively. Both patients remain in complete clinical and molecular remission without detectable *KIT* D816V mutation repeatedly performed up to 12 and 17 months after allogeneic SCT (data not shown).

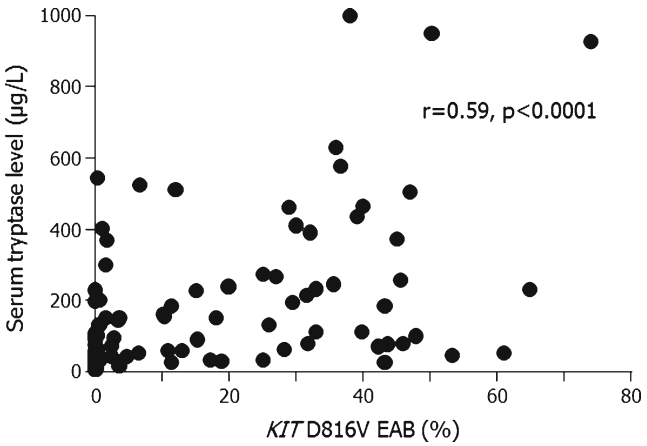

Fig. 2 Correlation of *KIT* D816V EAB with serum tryptase level (STL). Significant correlation between *KIT* D816V EAB in PB (x-axis) and STL (y-axis) irrespective of disease subtype

KIT D816V EAB and overall survival

The *KIT* D816V EAB is strongly correlated with disease activity (e.g., STL, $p<0.001$), disease subtype (e.g., indolent vs. advanced SM, $p<0.001$) and consequently survival. Any survival analysis based on different *KIT* D816V EAB levels, e.g., 0 vs. >0 %, <2 vs. ≥2 %, <5 vs. ≥5 %, <10 vs. ≥10 %, or <20 vs. ≥20 %, respectively, provides significant p values. The best overall discrimination is seen for the group 0 vs. >0 % ($p<0.0001$). None of the 57 ISM/SSM patients with a median *KIT* D816V EAB of 0.2 % has yet died, whereas 16 (median *KIT* D816V EAB of 36 %) of 67 (median *KIT* D816V EAB of 37 %) patients with advanced SM have died (data not shown).

Discussion

The accuracy and reliability with which cytogenetic and molecular aberrations are detected in hematologic malignancies are highly dependent on the number and source of cells as well as the techniques employed. Until recently, the most frequently used assay for the detection of point mutations and insertion/deletions has been CS of PCR-derived amplicons. However, the level of sensitivity is only between 10 and 20 %, good enough for detection of acute leukemias but leading to potentially false negative results in disorders with low disease burden, e.g., *JAK2* V617F-positive essential thrombocythemia or *KIT* D816V-positive ISM [23, 24].

While the source of cells is not important for most hematological malignancies at diagnosis, an exceptional situation is found in SM because mast cells usually entirely reside in the BM and are only very rarely found in PB [25, 26]. Consequently, it has been repeatedly suggested that BM is required for reliable detection of the *KIT* D816V mutation in SM. However, disease subtypes may greatly vary regarding

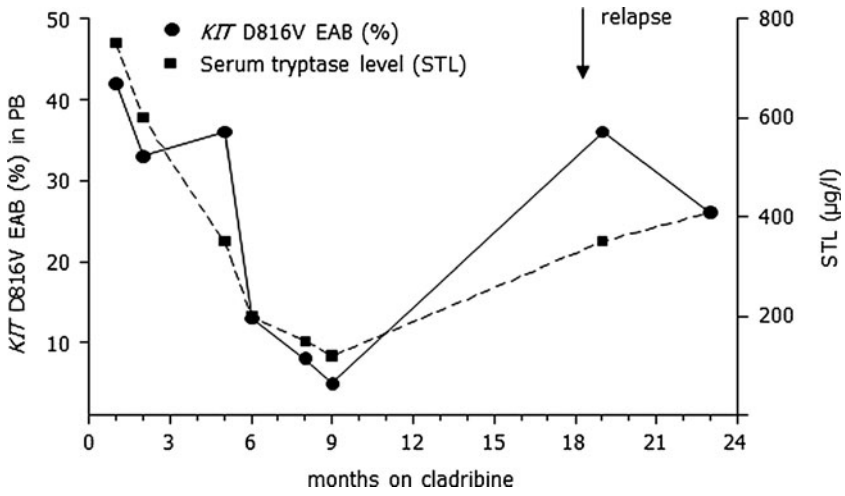

Fig. 3 *KIT* D816V EAB and STL during treatment with cladribine. Monitoring of the *KIT* D816V EAB in a patient with ASM-AHNMD during treatment with cladribine revealed a 1 log decrease from 42 to 5 %. The patient relapsed 18 months later, which was reflected by increased *KIT* D816V EAB and STL in PB

mast cell burden and involvement of other lineages. In addition, assay sensitivities have been considerably improved down to 0.1–0.01 % using genomic DNA based approaches, including PCR amplification followed by restriction digestion [27], peptide nucleic acid-mediated PCR clamping technique [11], and qualitative [28] and quantitative allele-specific PCR assays [20, 29]. More sophisticated techniques include cell sorting [30] or enrichment of malignant cells via laser microdissection followed by melting curve analysis of PCR products [31, 32].

We have established a novel allele-specific quantitative RT-PCR (RQ-PCR) with an improved sensitivity down to 0.01 % and consequently detected the *KIT* D816V mutation in 97 % of patients with histologically confirmed SM. Because the RQ-PCR assay cannot detect mutations other than D816V, we employed a D-HPLC assay in D816V-negative patients, which allowed the identification of alternative mutations within the PCR-generated amplicon. Four additional patients were identified with two *KIT* D816H and *KIT* D816Y mutations each, overall leaving only 1 out of 147 patients not carrying a *KIT* D816 mutation. In BM, positive results were obtained irrespective of disease subtype. All patients in advanced phase were also positive in PB potentially indicating involvement of several myeloid and/or lymphoid lineages as recently reported in patients with SSM [33] or ASM/MCL [11, 30]. In contrast, only 46 % of ISM patients were also positive in PB. Key factors for qualitative (positive/negative) and quantitative (*KIT* D816V EAB/genomic AB) presence of *KIT* D816V in PB therefore include disease burden, disease subtype, multilineage involvement, and assay sensitivity. Since the *KIT* D816V EAB is the basis of disease burden, we also observed a dependent correlation with disease subtype and consequently survival.

The broadest heterogeneity of *KIT* D816V EAB was observed in SSM and SM-AHNMD, although it still correlated strongly with STL. The presence or absence of *KIT* D816V in the cell compartment representing AHNMD remains elusive and requires elaborate molecular tests on sorted or microdissected cells [34]. In SM-CMML, monocytes are *KIT* D816V-positive in >90 % of cases, whereas the status of eosinophils in SM-HES/CEL remains less clear [11, 32]. In line with these results, we observed a significantly different *KIT* D816V EAB in patients with or without monocytosis but no differences in patients with or without eosinophilia. In contrast to ISM, it was suggested that in advanced SM the *KIT* mutation occurs in a multi progenitor cell resulting in multilineage involvement [35]. Consequently, a high level of *KIT* D816V mutation-positive progenitor cells were found in PB of advanced SM as compared to ISM and healthy controls [29, 36]. Of interest, we observed significant *KIT* D816V EAB differences between patients with ASM/MCL with or without AHNMD (38 vs. 21.5 %, $p=0.019$, data not shown).

With an assay sensitivity reported to down to 0.003 %, Kristensen et al. [29] detected the *KIT* D816V mutation in all 25 ISM patients tested. In four of these patients, the *KIT* D816V allele burden was <0.01 % in PB and <0.1 % in BM, respectively, which would not have allowed the detection of *KIT* D816V with our assay. One patient was diagnosed upon atypical morphology and positivity of CD2/CD25, while three patients fulfilled the major criterion of compact mast cell aggregates. Three ISM patients exhibited a genomic allele burden of more than 35 %, which we would only have observed in advanced disease. No detailed clinical data or STL were presented, which would have facilitated to explain potential differences of the study cohorts. Because the genomic assay has not yet been validated in patients with advanced disease, a direct comparison of both assays in 37 samples including all subtypes of SM was performed and provided a strong correlation. In patients with mutation levels below 0.1 %, the RQ-PCR is however less sensitive. Until recently, we have preferred RNA/cDNA for routine analysis because it also allows screening for fusion genes (e.g., *BCR-ABL1* and *FIP1L1-PDGFRA*) and gene expression levels (e.g., array technology, quantitative RT-PCR for expression of *PDGFRA*

and *PDGFRB*), particularly for patients with associated eosinophilia and monocytosis [37–39]. However, the recently shown importance of pathogenetically relevant, additional mutations, and the higher sensitivity of DNA assays in indolent disease may redirect the focus on analysis of DNA [21, 22]. In a small subset of ASM/MCL patients ($n=14$), we now have also shown the previously lacking potential value of this DNA assay in advanced phase disease. Of interest, all these studies share the common observation of disparities between BM mast cell infiltration, STL, genomic/transcriptional allele burden, and C findings. They may, at least in part, be explained by the recently described molecular heterogeneity of advanced SM with >50 % of patients being positive for four and more pathogenetically relevant point/length mutations [21].

RQ-PCR is a fast, sensitive, reliable, and cost-effective method for routine screening of *KIT* D816V in all SM subtypes. The *KIT* D816V EAB is strongly correlated with disease burden, disease subtype, and survival. PB is sufficient for mutation analysis in patients with advanced disease, while a negative result from PB should be complemented by a BM analysis in indolent SM. In the very rare event of negativity in histologically confirmed SM, patients should be screened by D-HPLC for rare mutations at codon 816, e.g., D816H or *KIT* D816Y, since they are not detected by allele-specific RQ-PCR. CS on unfractionated cells is certainly inadequate for reliable detection of *KIT* D816 mutations. Compared to established response markers like improvement of blood counts and reduction of organomegaly, BM mast cell infiltration, or STL, RQ-PCR is likely to provide most of its complimentary benefits at low levels of residual disease, e.g., after intensive chemotherapy or allogeneic SCT. Similar to CML, standardized RQ-PCR results may become objective and reproducible prognostic markers and treatment endpoints in clinical trials and daily clinical practice [40].

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References

1. Valent P, Horny HP, Escribano L, Longley BJ, Li CY, Schwartz LB et al (2001 Jul) Diagnostic criteria and classification of mastocytosis: a consensus proposal. Leuk Res 25(7):603–625
2. Horny HP, Sotlar K, Valent P (2007) Mastocytosis: state of the art. Pathobiology 74(2):121–132
3. Valent P, Akin C, Longley JB, Metcalfe DD, Parwaresch RM, Bennett JM (2001) Mastocytosis. In: Jaffe ES, NLH HS, Vardiman JW (eds) Pathology and genetics: tumours of haematopoietic and lymphoid tissues: World Health Organization (WHO) Classification of Tumours, vol 1. IARC, Lyon, pp 291–302
4. Horny HP, Valent P (2001 Jul) Diagnosis of mastocytosis: general histopathological aspects, morphological criteria, and immunohistochemical findings. Leuk Res 25(7):543–551
5. Tefferi A, Thiele J, Vardiman JW (2009 Sep 1) The 2008 World Health Organization classification system for myeloproliferative neoplasms: order out of chaos. Cancer 115(17):3842–3847
6. Valent P, Arock M, Akin C, Sperr WR, Reiter A, Sotlar K et al (2010 Aug 5) The classification of systemic mastocytosis should include mast cell leukemia (MCL) and systemic mastocytosis with a clonal hematologic non-mast cell lineage disease (SM-AHNMD). Blood 116(5):850–851
7. Vardiman JW (2010 Mar 19) The World Health Organization (WHO) classification of tumors of the hematopoietic and lymphoid tissues: an overview with emphasis on the myeloid neoplasms. Chem Biol Interact 184(1–2):16–20
8. Pardanani A (2012 Apr) Systemic mastocytosis in adults: 2012 Update on diagnosis, risk stratification, and management. Am J Hematol 87(4):401–411
9. Schittenhelm MM, Shiraga S, Schroeder A, Corbin AS, Griffith D, Lee FY et al (2006 Jan 1) Dasatinib (BMS-354825), a dual SRC/ABL kinase inhibitor, inhibits the kinase activity of wild-type, juxtamembrane, and activation loop mutant KIT isoforms associated with human malignancies. Cancer Res 66(1):473–481
10. Valent P, Akin C, Sperr WR, Mayerhofer M, Fodinger M, Fritsche-Polanz R et al (2005 Jan) Mastocytosis: pathology, genetics, and current options for therapy. Leuk Lymphoma 46(1):35–48
11. Garcia-Montero AC, Jara-Acevedo M, Teodosio C, Sanchez ML, Nunez R, Prados A et al (2006 Oct 1) KIT mutation in mast cells and other bone marrow hematopoietic cell lineages in systemic mast cell disorders: a prospective study of the Spanish Network on Mastocytosis (REMA) in a series of 113 patients. Blood 108(7):2366–2372
12. Valent P, Akin C, Escribano L, Fodinger M, Hartmann K, Brockow K et al (2007 Jun) Standards and standardization in mastocytosis: consensus statements on diagnostics, treatment recommendations and response criteria. Eur J Clin Invest 37(6):435–453
13. Akin C, Metcalfe DD (2004 Jul) The biology of Kit in disease and the application of pharmacogenetics. J Allergy Clin Immunol 114(1):13–19, quiz 20
14. Pardanani A, Reeder T, Li CY, Tefferi A (2003 Oct) Eosinophils are derived from the neoplastic clone in patients with systemic mastocytosis and eosinophilia. Leuk Res 27(10):883–885
15. Taylor ML, Sehgal D, Raffeld M, Obiakor H, Akin C, Mage RG et al (2004 Nov) Demonstration that mast cells, T cells, and B cells bearing the activating kit mutation D816V occur in clusters within the marrow of patients with mastocytosis. J Mol Diagn 6(4):335–342
16. Yavuz AS, Lipsky PE, Yavuz S, Metcalfe DD, Akin C (2002 Jul 15) Evidence for the involvement of a hematopoietic progenitor cell in systemic mastocytosis from single-cell analysis of mutations in the c-kit gene. Blood 100(2):661–665
17. Butterfield JH, Weiler D, Dewald G, Gleich GJ (1988) Establishment of an immature mast cell line from a patient with mast cell leukemia. Leuk Res 12(4):345–355
18. Cross NC, Hughes TP, Feng L, O'Shea P, Bungey J, Marks DI et al (1993 May) Minimal residual disease after allogeneic bone marrow transplantation for chronic myeloid leukaemia in first chronic phase: correlations with acute graft-versus-host disease and relapse. Br J Haematol 84(1):67–74
19. Muller MC, Erben P, Saglio G, Gottardi E, Nyvold CG, Schenk T et al (2008 Jan) Harmonization of BCR-ABL mRNA quantification

using a uniform multifunctional control plasmid in 37 international laboratories. Leukemia 22(1):96–102
20. Kristensen T, Vestergaard H, Moller MB (2011 Mar) Improved detection of the KIT D816V mutation in patients with systemic mastocytosis using a quantitative and highly sensitive real-time qPCR assay. J Mol Diagn 13(2):180–188
21. Schwaab J, Schnittger S, Sotlar K, Walz C, Fabarius A (2013 Aug 19) Pfirrmann M, et al. Comprehensive mutational profiling in advanced systemic mastocytosis, Blood
22. Traina F, Visconte V, Jankowska AM, Makishima H, O'Keefe CL, Elson P et al (2012) Single nucleotide polymorphism array lesions, TET2, DNMT3A, ASXL1 and CBL mutations are present in systemic mastocytosis. PLoS One 7(8):e43090
23. Corless CL, Harrell P, Lacouture M, Bainbridge T, Le C, Gatter K et al (2006 Nov) Allele-specific polymerase chain reaction for the imatinib-resistant KIT D816V and D816F mutations in mastocytosis and acute myelogenous leukemia. J Mol Diagn 8(5):604–612
24. Lippert E, Boissinot M, Kralovics R, Girodon F, Dobo I, Praloran V et al (2006 Sep 15) The JAK2-V617F mutation is frequently present at diagnosis in patients with essential thrombocythemia and polycythemia vera. Blood 108(6):1865–1867
25. Galli SJ (2000 Jan) Mast cells and basophils. Curr Opin Hematol 7(1):32–39
26. Galli SJ (1990 Jan) New insights into "the riddle of the mast cells": microenvironmental regulation of mast cell development and phenotypic heterogeneity. Lab Invest 62(1):5–33
27. Tan A, Westerman D, McArthur GA, Lynch K, Waring P, Dobrovic A (2006 Dec) Sensitive detection of KIT D816V in patients with mastocytosis. Clin Chem 52(12):2250–2257
28. Schumacher JA, Elenitoba-Johnson KS, Lim MS (2008 Jan) Detection of the c-kit D816V mutation in systemic mastocytosis by allele-specific PCR. J Clin Pathol 61(1):109–114
29. Kristensen T, Broesby-Olsen S, Vestergaard H, Bindslev-Jensen C, Moller MB (2012 Jul) Circulating KIT D816V mutation-positive non-mast cells in peripheral blood are characteristic of indolent systemic mastocytosis. Eur J Haematol 89(1):42–46
30. Teodosio C, Garcia-Montero AC, Jara-Acevedo M, Alvarez-Twose I, Sanchez-Munoz L, Almeida J et al (2012 May) An immature immunophenotype of bone marrow mast cells predicts for multilineage D816V KIT mutation in systemic mastocytosis. Leukemia 26(5):951–958
31. Noack F, Escribano L, Sotlar K, Nunez R, Schuetze K, Valent P et al (2003 Feb) Evolution of urticaria pigmentosa into indolent systemic mastocytosis: abnormal immunophenotype of mast cells without evidence of c-kit mutation ASP-816-VAL. Leuk Lymphoma 44(2): 313–319
32. Sotlar K, Fridrich C, Mall A, Jaussi R, Bultmann B, Valent P et al (2002 Nov) Detection of c-kit point mutation Asp-816 –>Val in microdissected pooled single mast cells and leukemic cells in a patient with systemic mastocytosis and concomitant chronic myelomonocytic leukemia. Leuk Res 26(11):979–984
33. Valent P, Akin C, Sperr WR, Horny HP, Metcalfe DD (2002 Feb) Smouldering mastocytosis: a novel subtype of systemic mastocytosis with slow progression. Int Arch Allergy Immunol 127(2):137–139
34. Sotlar K, Colak S, Bache A, Berezowska S, Krokowski M, Bultmann B et al (2010 Apr) Variable presence of KITD816V in clonal haematological non-mast cell lineage diseases associated with systemic mastocytosis (SM-AHNMD). J Pathol 220(5):586–595
35. Akin C (2005) Clonality and molecular pathogenesis of mastocytosis. Acta Haematol 114(1):61–69
36. Georgin-Lavialle S, Lhermitte L, Baude C, Barete S, Bruneau J, Launay JM et al (2011 Nov 10) Blood CD34-c-Kit+cell rate correlates with aggressive forms of systemic mastocytosis and behaves like a mast cell precursor. Blood 118(19):5246–5249
37. Emig M, Saussele S, Wittor H, Weisser A, Reiter A, Willer A et al (1999 Nov) Accurate and rapid analysis of residual disease in patients with CML using specific fluorescent hybridization probes for real time quantitative RT-PCR. Leukemia 13(11):1825–1832
38. Cools J (2005) FIP1L1-PDGFR alpha, a therapeutic target for the treatment of chronic eosinophilic leukemia. Verh K Acad Geneeskd Belg 67(3):169–176
39. Erben P, Gosenca D, Muller MC, Reinhard J, Score J, Del Valle F et al (2010 May) Screening for diverse PDGFRA or PDGFRB fusion genes is facilitated by generic quantitative reverse transcriptase polymerase chain reaction analysis. Haematologica 95(5):738–744
40. Pardanani A, Tefferi A (2010 May) A critical reappraisal of treatment response criteria in systemic mastocytosis and a proposal for revisions. Eur J Haematol 84(5):371–378